Kinetic Study of the Effect of Metabisulfite on Polyphenol Oxidase

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A kinetic study of the inhibition of the catecholase activity of polyphenol oxidase by metabisulfite has been made. Metabisulfite affected enzymatic browning in two different ways: (a) it reacted with the quinones produced by catalytic activity, and (b) it had a direct effect on the enzyme by irreversibly binding to both "met" and "oxy" forms of the enzyme, with different inactivation rate constants. Kinetic data obtained by varying the substrate concentration showed a positive kinetic cooperativity effect. These results indicate that metabisulfite is an example of an irreversible enzyme inhibitor rendered unstable in the reaction medium by enzymatic catalysis. The kinetic model proposed for its action mechanism was simulated in the computer, and a good agreement with the experimental results was obtained.

INTRODUCTION

The undesirable browning of fruits and vegetables is caused mainly by the oxidation of phenols in the tissue by polyphenol oxidase (EC 1.14.18.1). This enzyme is a multifunctional copper-containing protein that catalyzes both the orthohydroxylation of monophenols to *o*-diphenols (cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (catecholase activity). Three different forms of binuclear copper in the active involved in the reaction mechanism are known: met, oxy, and deoxy (Lerch, 1981; Robb, 1981). The quinones formed react with themselves and also with amino acids or proteins to give the characteristic brown pigments (Mason and Peterson, 1965; Matheis and Whitaker, 1984; García-Carmona et al., 1988). This phenomenon is therefore of great organoleptic and nutritional importance in food processing.

Sulfite is one of the most widely used inhibitors of enzymatic browning in food technology and appears to be the most practical oxidation inhibitor in the wine industry (Ough and Crowell, 1987). Nowadays, however, because of greater awareness of its possible damaging effect to health, there is a tendency to reduce, if not eliminate, its use. Thus, several studies have been performed in which wines were prepared with various sulfite levels, the observed differences between some physicochemical parameters related to enzymatic browning being measured throughout the vinification process (Kidron et al., 1978; Wissemann and Lee, 1980; Valero et al., 1989). However, it is also necessary to increase our knowledge of the action mechanism of sulfite in enzymatic browning, so that alternatives may be suggested.

The effect of sulfite on polyphenol oxidase is complex and has caused much controversy over the years. It has been shown to react with the quinones produced by enzymatic activity and reduce them back to their corresponding *o*-diphenolic substrates (Walker, 1975), although evidence concerning the formation of colorless addition products has also been found (Embs and Markakis, 1965). The observed loss of catalytic activity has been attributed to the suicide inactivation of the enzyme (Golan-Goldhirsh and Whitaker, 1985), and it has also been shown that sulfite has a direct effect on the enzyme (Golan-Goldhirsh and Whitaker, 1984; Sayavedra-Soto and Montgomery, 1986). In spite of all this, there is still little information in the literature about the mechanism of polyphenol oxidase inhibition by sulfite at the molecular level, perhaps because of the complexity of the former's internal catalytic mechanism.

In the present paper, a kinetic analysis of the inhibition mechanism of the catecholase activity of polyphenol oxidase caused by metabisulfite has been made. Likewise, computer simulations of its behavior are presented, and the kinetic parameters and constants characterizing the system have been evaluated.

MATERIALS AND METHODS

Plant Material. The grapes (*Vitis vinifera* L. cv. Airen) used in this study were harvested at maturation stage in Villarrobledo (Albacete, Spain) and stored at −25 °C until use.

Reagents. 4-Methylcatechol (4MC) and ascorbic acid were purchased from Sigma Chemical Co., and sodium metabisulfite was purchased from Merck. Fresh solutions of these reactives were prepared every day. Other reagents were all of analytical grade.

Methods. Polyphenol oxidase was extracted from grapes as previously described (Valero et al., 1988a).

Catecholase activity of polyphenol oxidase was followed by observing the appearance of 4-methyl-*o*-benzoquinone at 400 nm since it has recently been established that the *o*-quinone has a half-life of 1400 s (Valero et al., 1988b). One unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol of 4-methyl-*o*-benzoquinone/min. Unless otherwise stated, the reaction media at 25 °C contained 30 mM 4-methylcatechol, 10 mM sodium acetate buffer (pH 4.75), metabisulfite at the indicated concentration, and 0.15 unit of enzymatic activity. Protein concentration was determined according to the method of Bradford (1976). Steady-state rate (V_s) was defined as the slope of the linear zone of the product accumulation curve. The lag period (L) was evaluated by extrapolation of the linear portion of the product accumulation curve to the abscissa axis.

Inactivation of polyphenol oxidase by metabisulfite in the absence of substrate was performed by preincubating the enzyme (26 μg) at 25 °C with 100 mM sodium acetate buffer (pH 4.75) and the indicated concentrations of inhibitor, in a final volume of 500 μL. Aliquots (30 μL) were withdrawn at various times, and catecholase activity was assayed immediately in the standard conditions in the absence of inhibitor. Activities at time zero were taken as 100%.

Computer simulations were performed according to the numerical integration method of Euler, introduced in a compiled

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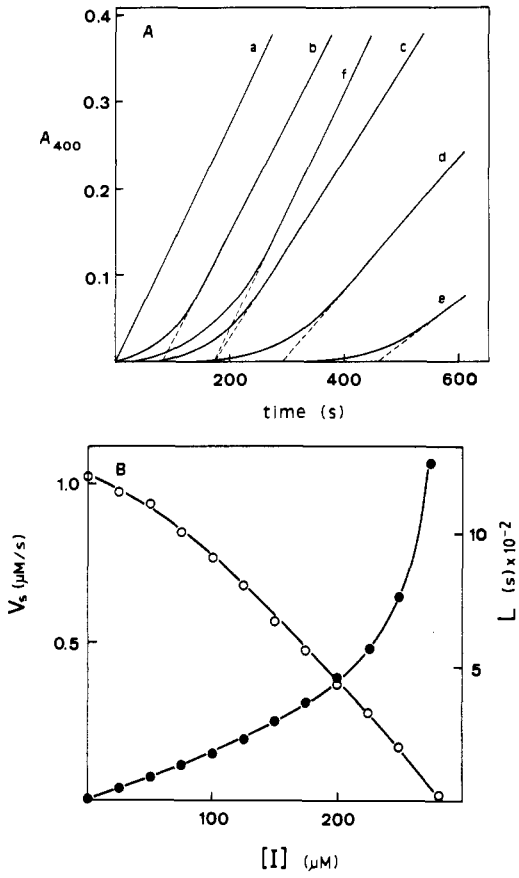


Figure 1. (A) Time course of catecholase activity of polyphenol oxidase in the presence of metabisulfite (curves a–e) and ascorbic acid (curve f). Metabisulfite concentrations used were (a) 0, (b) 50, (c) 100, (d) 150, and (e) 200 μM. Ascorbic acid concentration used was 175 μM. (B) Effect of metabisulfite concentration on lag period (L) (●) and on enzyme reaction velocity at steady state (V_s) (○).

BASIC program using an IBM PC-XT microcomputer equipped with math coprocessor. The integration step varied automatically in the program by means of a 1–10% flow tolerance method (Barshop et al., 1983). The kinetic parameters V_m and K_M corresponding to 4-methylcatechol were determined by fitting the experimental data obtained in the absence of inhibitor to the Michaelis–Menten equation using nonlinear regression (Marquardt, 1963). Values for the unknown constants were chosen in such a way that the model produced reasonable rates under the simulation conditions.

RESULTS AND DISCUSSION

The presence of metabisulfite in the reaction medium modifies the time course of the catecholase activity of polyphenol oxidase when followed spectrophotometrically. Figure 1A shows a selection of progress curves obtained in the presence and absence of metabisulfite, the shape of each curve (product accumulation curve) in the presence of inhibitor including (a) an initial lag period due to the chemical reactions between the quinone product of the enzymatic catalysis and metabisulfite and (b) a steady-state phase, where all of the inhibitor has been depleted and during which the catalytic activity expressed is lower than in the absence of metabisulfite.

The dependence of these two kinetic parameters, L and V_s, on metabisulfite concentration is shown in Figure 1B. It can be seen that the duration of the lag period is not linearly dependent on metabisulfite concentration. This, together with the observed inhibition of activity, may be due to the direct effect of metabisulfite on polyphenol oxidase (Golan-Goldhirsh and Whitaker, 1984; Sayavedra-Soto and Montgomery, 1986), although irreversible inactivation by enzymatic catalysis (suicide inactivation), as has been shown with mushroom (Varoquaux and Sarris, 1979; Golan-Goldhirsh and Whitaker, 1985; Tudela et al., 1987) and frog epidermis polyphenol oxidase (Escribano et al., 1989), as well as substrate depletion may also contribute to it. To check these two latter effects, we performed similar experiments in the presence of ascorbic acid instead of metabisulfite, since it acts by an analogous mechanism and has been shown to have no direct effect on polyphenol oxidase (Varoquaux and Sarris, 1979). The postlag period rates observed were not affected by the presence of ascorbic acid in concentrations that produced lag periods similar to those obtained with metabisulfite (Figure 1A, curve f). By this means, we were able to discount the existence of suicide inactivation as well as substrate depletion throughout the measurement time. This indicated that the length of the lag period reflects both the enzyme inactivation rate and the chemical reaction rate between metabisulfite and the quinone product of enzymatic activity. Once all of the metabisulfite has been depleted, the steady-state rate observed reflects the amount of active enzyme remaining. The duration of the lag period tends to infinity at a metabisulfite concentration which causes the complete inhibition of the enzyme, so that the inhibitor is never totally consumed.

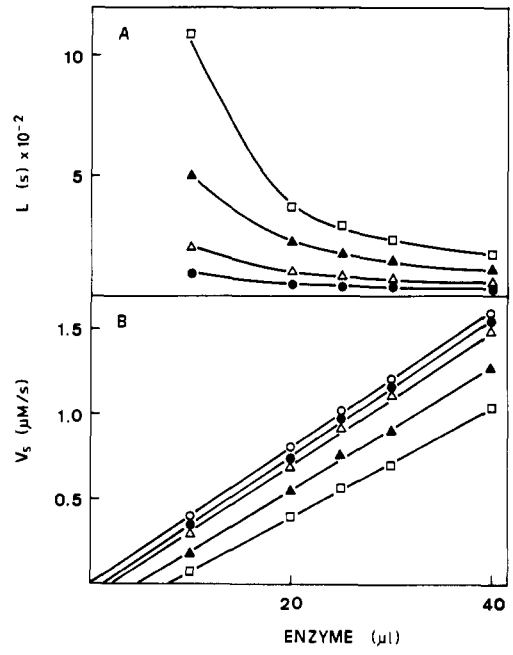


Figure 2. Effect of polyphenol oxidase concentration on L (A) and V_s (B) at different initial metabisulfite concentrations. Metabisulfite concentrations used were (○) 0, (●) 25, (△) 50, (▲) 100, and (□) 150 μM.

Figures 2 and 3, respectively, show the effect of enzyme and substrate concentrations on lag period and steady-state rate at various initial concentrations of metabisulfite. It can be seen that the length of the lag period diminishes with both enzyme and substrate concentrations (Figures 2A and 3A), this diminution being even more pronounced at low concentrations. With respect to the catalytic activity expressed when the steady state is reached, dependence upon enzyme concentration (Figure 2B) shows an irreversible type of inhibition since straight lines do not pass through the coordinate origin (Segel, 1975). On the other hand, the fact that double-reciprocal plots with respect to substrate concentration were not linear (Figure 3B) reveals that there is a cooperative effect in the system (Neet, 1980). To examine this effect, we fit these data using nonlinear

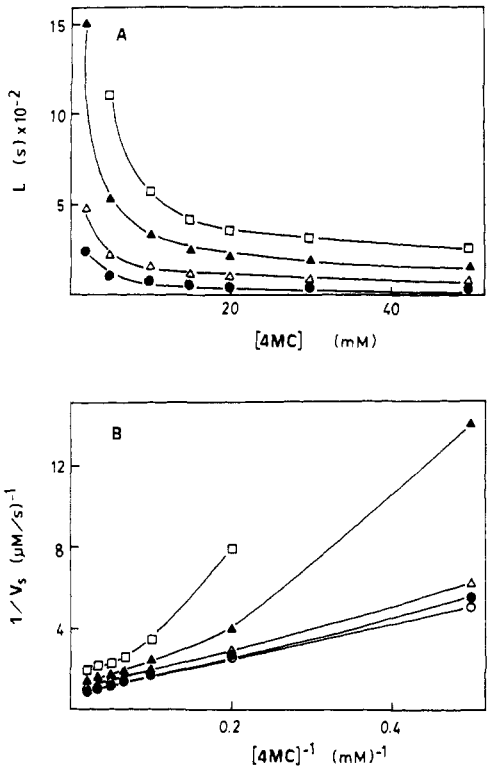


Figure 3. Effect of 4-methylcatechol concentration on L (A) and V_s (B) at different initial metabisulfite concentrations. Conditions were as in Figure 2.

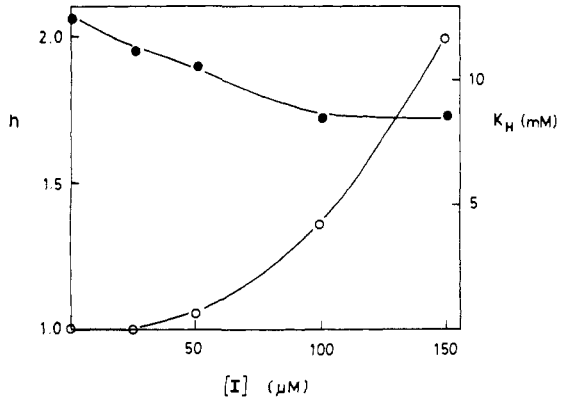


Figure 4. Variation of the Hill coefficient (○) and constant of the Hill equation (●) with metabisulfite concentration.

regression (Marquardt, 1963) to the Hill equation

$$v = V_m[S]^h/(K_H^h + [S]^h) \quad (1)$$

where h is the Hill coefficient, widely used as an index of cooperativity, and K_H is known as the constant of the Hill equation. The results obtained are shown in Figure 4, positive kinetic cooperativity ($h > 1$) being obtained in the presence of metabisulfite. This response of the system is similar to the one obtained by computer simulation of the behavior of an irreversible enzyme inhibitor rendered unstable in the reaction medium by enzymatic catalysis as shown by the inhibition of polyphenol oxidase by L-cysteine (Valero et al., 1991). This permits a kinetic scheme for the metabisulfite action mechanism on enzymatic browning to be set up.

To test the applicability of this model to the action mechanism of metabisulfite, it was necessary to study its direct inhibitory effect on polyphenol oxidase in depth. Thus, several experiments were performed, in which similar levels of enzyme activity were exposed to increasing levels of

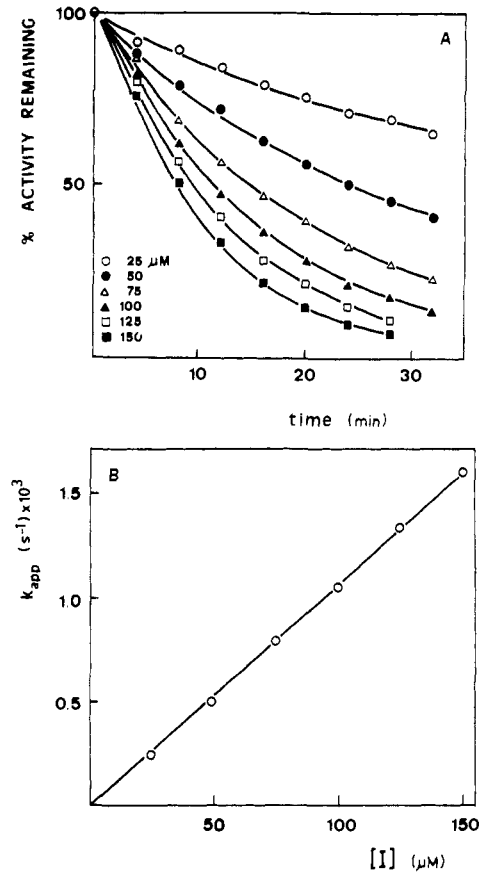


Figure 5. (A) Inactivation of grape polyphenol oxidase by metabisulfite. (B) Apparent inactivation constants of the free (met) form of the enzyme in the absence of substrate, as a function of metabisulfite concentrations.

Table I. Comparison of Postlag Period Activities Obtained in a Normal Assay in the Presence of Metabisulfite (V_1) and after Polyphenol Oxidase Was Preincubated with This Inhibitor (V_2) for a Length of Time Equal to the Lag Period (L) Obtained in a Normal Assay at Each Metabisulfite Concentration

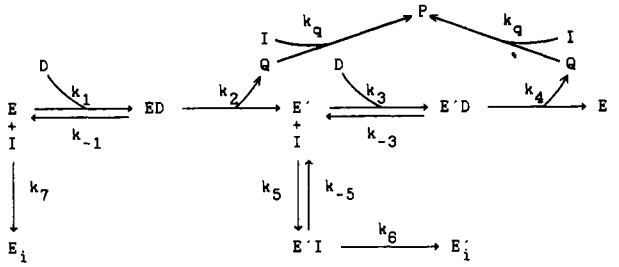
[I], μM	V_1, μM/s	L, s	V_2,[a] μM/s
50	0.88	78.2	0.97
75	0.81	117.6	0.92
100	0.71	168.5	0.85
125	0.61	228.3	0.75
150	0.49	292.0	0.72
175	0.40	368.5	0.67
200	0.32	473.8	0.59
225	0.23	624.4	0.52
250	0.14	768.0	0.46

[a] Preincubation mixtures were as described under Materials and Methods.

metabisulfite and incubated at the previously mentioned pH and temperature conditions, assaying the catecholase activity remaining at various times (Figure 5A). To establish the reaction order and to determine the inactivation process constant, replots were made of these data [ln percent activity remaining vs time (data not shown), and the slope from each straight line thus obtained vs inhibitor concentration (Figure 5B)]. The fact that these plots were linear indicated that these data fit the equation corresponding to a first-order process ($[E] = [E]_0e^{-k_{app}t}$, where $k_{app} = k[I]$); that is to say, the inactivation of free enzyme by metabisulfite proceeds kinetically in only one step ($E + I \rightarrow_{(k)} E_i$), with an inactivation constant rate of 10.35 M^{-1} s^{-1}.

However, it follows from Figure 5 that the degree of inactivation obtained by preincubating polyphenol oxi-

Scheme I. Proposed Mechanism for the Inhibition of Catecholase Activity of Polyphenol Oxidase by Metabisulfite[a]



[a] E and E′, met and oxy forms of polyphenol oxidase, respectively; D, orthodiphenolic substrate; P, product of the reaction between metabisulfite (I) and the quinone (Q) product of catalytic activity. P may be a substrate of the enzyme.

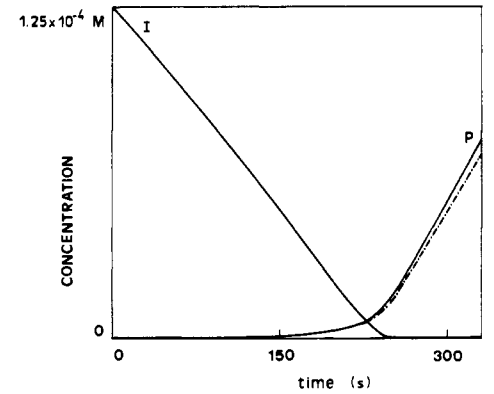


Figure 6. Progress curves for the inhibition of polyphenol oxidase by metabisulfite (125 μM) obtained experimentally (- · -) and by computer simulation of Scheme I (eqs 12–14) (—). The following kinetic constants were used: $K_M = 1.25 \times 10^{-2}$ M; $k_{cat} = 1.45 \times 10^3$ s^{-1}; $K_I = 3 \times 10^{-5}$ M; $k_6 = 4.15 \times 10^{-3}$ s^{-1}; $k_i = 8.3 \times 10^{-4}$ M^{-1} s^{-1}; $k_q = 4 \times 10^3$ M^{-1} s^{-1}. Initial conditions were as follows: $[S]_0 = 3 \times 10^{-2}$ M; $[Q]_0 = 0$; $[I]_0 = 1.25 \times 10^{-4}$ M; $[E_s]_0 = 10^{-9}$ M.

dase with metabisulfite in the absence of substrate is lower than that obtained in its presence, when the reaction is started by the addition of the nonpreincubated enzyme (Figure 1). This fact was confirmed by performing new experiments in which polyphenol oxidase was preincubated with metabisulfite for a length of time equal to the lag period obtained in a normal assay in the presence of the same concentration of metabisulfite (Table I). This result can only be interpreted by taking into account the internal mechanism of catecholase activity of polyphenol oxidase (Galindo et al., 1983; Lerch, 1983; Cabanes et al., 1987), revealing that metabisulfite irreversibly binds to both "met" and "oxy" forms of the enzyme involved in their internal catalytic mechanism, although with different inactivation rate constants. On this basis, the mechanism for the inhibition of the catecholase activity of polyphenol oxidase by metabisulfite could be written as shown in Scheme I. It is assumed that the inactivation of the oxy form of polyphenol oxidase (E′) involves the formation of an enzyme–inhibitor complex (E′I), which subsequently undergoes an irreversible isomerization reaction to give the inactive enzyme (E'_i). This seems preferable to a one-step inactivation since the corresponding data obtained by computer simulation (see below) agreed better with the experimental results.

The differential equation system corresponding to this kinetic scheme was simplified by following the method of Cha (1968) and bearing in mind similar assumptions to those proposed for a general case (Valero et al., 1991); the following set of equations was obtained:

$$d[E_s]/dt = -(k_7 f_E[I] + k_6 f_{E'I})[E_s] \quad (2)$$

$$d[Q]/dt = (k_2 f_{ED} + k_4 f_{E'D})[E_s] - k_q[I][Q] \quad (3)$$

$$d[I]/dt = -k_q[I][Q] \quad (4)$$

Here E_s is the group of the enzyme species involved in a restricted steady state ($[E_s] = [E] + [ED] + [E'] + [E'D] + [E'I]$) and f_x is the concentration factor corresponding to enzyme species X with regard to E_s ($f_x = [X]/[E_s]$).

$$f_E = \frac{K_M k_i}{[D] + K_M\left(1 + \frac{[I]}{K_I}\right)} \quad (5)$$

$$f_{E'I} = \frac{k_6 K_M/K_I}{[D] + K_M\left(1 + \frac{[I]}{K_I}\right)} \quad (6)$$

$$f_{ED} = \frac{k_4[D]/(k_2 + k_4)}{[D] + K_M\left(1 + \frac{[I]}{K_I}\right)} \quad (7)$$

$$f_{E'D} = \frac{k_2[D]/(k_2 + k_4)}{[D] + K_M\left(1 + \frac{[I]}{K_I}\right)} \quad (8)$$

Here K_M and K_I are the apparent Michaelis–Menten constant for the orthodiphenolic substrate and the apparent dissociation constant of the E′I complex, respectively, whose expressions are the following:

$$K_M = \frac{k_1k_2(k_{-3} + k_4) + k_3k_4(k_{-1} + k_2)}{k_1k_3(k_2 + k_4)} \quad (9)$$

$$K_I = \frac{k_{-5}[k_1k_2(k_{-3} + k_4) + k_3k_4(k_{-1} + k_2)]}{k_1k_2k_5(k_{-3} + k_4)} \quad (10)$$

k_i is the apparent constant of the met form of polyphenol oxidase inactivation by metasulfite in the presence of the orthodiphenolic substrate and is equal to

$$k_i = \frac{k_3k_4k_7(k_{-1} + k_2)}{k_3k_4(k_{-1} + k_2) + k_1k_2(k_{-3} + k_4)} \quad (11)$$

Inserting expressions 5 and 6 and 7 and 8 into eqs 2 and 3, respectively, we obtain the following differential equation system:

$$\frac{d[E_s]}{dt} = -[I][E_s]\frac{K_M(k_i + k_6/K_I)}{[D] + K_M\left(1 + \frac{[I]}{K_I}\right)} \quad (12)$$

$$\frac{d[Q]}{dt} = \frac{k_{cat}[D][E_s]}{[D] + K_M\left(1 + \frac{[I]}{K_I}\right)} - k_q[I][Q] \quad (13)$$

$$d[I]/dt = -k_q[I][Q] \quad (14)$$

Here k_{cat} is the catalytic constant of the catalytic process

and is given by

$$k_{cat} = 2k_2k_4/(k_2 + k_4) \quad (15)$$

This differential equation system (eqs 12–14) is not linear, and particular solutions were obtained by means of numerical integration as described under Materials and Methods, the initial conditions being $[E_s] = E_0$, $[I] = I_0$, and $[Q] = 0$. The results obtained when the time course of the reaction was simulated are shown in Figure 6, and a good agreement with the experimental results can be observed. By this means, we can obtain the dependence of the lag period and steady-state rate on inhibitor, enzyme, and substrate concentrations. These results are very similar to those of the experimental results (Figures 1B, 2, and 3) and support the kinetic model proposed in this work for the action mechanism of metabisulfite on polyphenol oxidase.

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